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# Coexistence of Multiple Multilocus Variable-Number Tandem-Repeat Analysis Subtypes of *Clostridium difficile* PCR Ribotype 027 Strains within Fecal Specimens▽

Hannah E. Tanner,[1]* Katherine J. Hardy,[1,2] and Peter M. Hawkey[1,2]

*Health Protection Agency, West Midlands Public Health Laboratory, Heart of England NHS Foundation Trust, Birmingham B9 5SS, United Kingdom,[1] and School of Infection and Immunity, University of Birmingham, Birmingham B15 2TT, United Kingdom[2]*

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**We investigated whether multilocus variable-number tandem-repeat analysis (MLVA) typing could identify different subtypes of *Clostridium difficile* ribotype 027 within the same feces specimen. Five of 39 specimens yielded at least one isolate with an MLVA profile different (more than five summed tandem repeat differences) from that of other isolates in the same specimen, thereby potentially obscuring epidemiological links between *C. difficile* infection cases.**

The incidence and severity of *Clostridium difficile* infection (CDI) have increased in recent years, possibly due to the emergence and spread of epidemic strain PCR ribotype 027 (also known as pulsed-field electrophoresis type NAP1) (9, 10, 12). Ribotype 027 is highly prevalent in the United Kingdom (2), making PCR ribotyping alone insufficient for investigating potential cases of cross infection or differentiating between epidemic outbreaks. Multilocus variable-number tandem-repeat analysis (MLVA) is a more discriminatory typing technique for *C. difficile* (11, 14) and has been used for several studies (1, 4–7), but as yet, no data on the subtyping of multiple isolates from the same specimen have been published.

We investigated whether MLVA typing could identify different subtypes of ribotype 027 within the same specimen and considered the impact this may have on the utility of MLVA as a typing method for outbreak situations.

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Thirty-nine feces samples (preselected by infection control teams on the basis of severity, clusters, or high CDI rates) submitted for PCR ribotyping and yielding a type 027 *C. difficile* isolate were arbitrarily selected. *C. difficile* was cultured from specimens using alcohol shock and inoculation onto cycloserine-cefoxitin-fructose agar. Five isolated colonies of *C. difficile* were picked from each culture and were all confirmed as *C. difficile* type 027 by PCR ribotyping (13), giving 195 study isolates.

Six variable-number tandem-repeat (VNTR) loci (A6Cd, B7Cd, C6Cd, E7Cd, G8Cd, and CDR60) from published MLVA schemes (11, 14) were amplified by PCR from all isolates. Loci F3Cd and H9Cd (14) were not used, as they are invariant within PCR ribotype 027 isolates (6, 8, 14). Loci CDR5 and CD59 (11) were not used, as they were found to be variant by only one tandem repeat or invariant in diverse 027 isolates (data not shown). Primers were used as previously described (14), except for the G8Cd reverse primer (5′ AAT CTAATAATCCAGTAATTTAAATT 3′), which was redesigned to improve the yield of G8Cd, and CDR60 primers (CDR60-Forward, 5′-AGTTTGTAGGGAAGTGTGTAAAT AGAT-3′; CDR60-Reverse, 5′-CGCATTAAATTTCACTCC TCAT-3′), which were redesigned to minimize the PCR product size. Five-microliter volumes of DNA extracts were added to 20 μl of PCR mixture, giving (final concentrations) 0.2 μM each primer, 2.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 1× GeneAmp PCR Gold buffer, and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). Reaction mixtures underwent activation at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min with a final elongation at 72°C for 5 min. PCR products were electrophoresed on 3% MetaPhor agarose (Lonza) gels in 0.5× Tris-borate-EDTA buffer at 150 V for 5 h against a 20-bp molecular size standard (Sigma-Aldrich Company Ltd.). Gels were stained with ethidium bromide and photographed under UV light. PCR product sizes were determined using BioNumerics software (Applied Maths) by comparison with a standard curve generated from the 20-bp ladder. VNTR numbers were calculated from PCR product sizes. Tandem-repeat numbers from each VNTR locus were concatenated for each isolate to form MLVA profiles, which were compared using BioNumerics software (Applied Maths).

The accuracy of determining VNTR numbers using the agarose gel method described was validated by sequencing 35 different VNTR PCR products using an ABI 3700 capillary sequencer to determine exact repeat numbers and then comparing them with numbers determined by the agarose gel method. A range of PCR product sizes from each locus including PCR products differing in size by one 6-bp repeat were included. The agarose gel method was accurate for 28 of 35 sequenced products with an error of plus or minus one repeat in the remaining seven. The impact of any such error was minimized by ensuring that PCR

* Corresponding author. Mailing address: Health Protection Agency, West Midlands Public Health Laboratory, Heart of England NHS Foundation Trust, Birmingham B9 5SS, United Kingdom. Phone: 44 (0) 121 424 2465. Fax: 44 (0) 121 772 6229. E-mail: Hannah.Tanner@HeartofEngland.nhs.uk.

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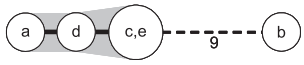
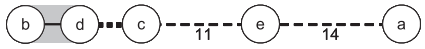
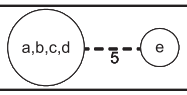
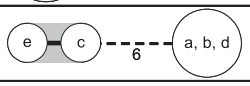
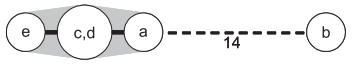
| Specimen | Isolate | MLVA Profile* | | | | | | Minimum Spanning Trees |
|---|---|---|---|---|---|---|---|---|
| | | A6Cd | B7Cd | C6Cd | E7Cd | G8Cd | CDR60 | |
| 1 | a | 39 | 18 | 45 | 10 | 15 | 10 |  |
| | b | 37 | 17 | 53 | 10 | 15 | 10 | |
| | c, e | 38 | 18 | 46 | 10 | 15 | 10 | |
| | d | 38 | 18 | 45 | 10 | 15 | 10 | |
| 3 | a | 18 | 7 | 17 | 12 | 15 | 10 |  |
| | b | 41 | 8 | 17 | 12 | 15 | 10 | |
| | c | 40 | 7 | 17 | 12 | 16 | 10 | |
| | d | 42 | 7 | 17 | 12 | 15 | 10 | |
| | e | 31 | 7 | 18 | 12 | 15 | 10 | |
| 10 | a, b, c, d | 38 | 20 | 19 | 12 | 15 | 10 |  |
| | e | 33 | 20 | 19 | 12 | 15 | 10 | |
| 12 | a, b, d | 38 | 22 | 21 | 12 | 15 | 10 |  |
| | c | 32 | 22 | 21 | 12 | 15 | 10 | |
| | e | 32 | 22 | 22 | 12 | 15 | 10 | |
| 15 | a | 38 | 20 | 21 | 12 | 16 | 10 |  |
| | b | 24 | 20 | 21 | 12 | 16 | 10 | |
| | c, d | 39 | 20 | 21 | 12 | 16 | 10 | |
| | e | 40 | 20 | 21 | 12 | 16 | 10 | |

*Numerical values in the table refer to the number of repeats for each VNTR locus

FIG. 1. MLVA profiles and minimum spanning trees for specimens yielding *C. difficile* PCR ribotype 027 isolates at least one of which had an STRD of five or more from the next most closely related isolate. For minimum spanning trees, circles represent unique MLVA profiles in the tree and are scaled by member count. Thick solid lines represent an STRD of one, thin solid lines represent an STRD of two, thick dashed lines represent an STRD of three, and thinner dashed lines represent an STRD of four or more. STRDs of more than four are indicated by numerals between the circles. Gray shading indicates complexes with a maximum neighbor distance of two tandem repeats and a minimum of two MLVA types.

products from the same locus for isolates from the same specimen were electrophoresed in adjacent lanes of the gel, making the smallest 6-bp differences clearly visible. Thus, any error was unlikely to affect the calculated differences between isolates. Accurate size determination methods such as sequencing would be required for larger-scale comparisons.

Studies using *C. difficile* MLVA (1, 4, 5, 7) have utilized the Manhattan coefficient to calculate a summed tandem-repeat

TABLE 1. MLVA profiles of specimens containing *C. difficile* PCR ribotype 027 isolates differing from each other at three out of six VNTR loci

| Specimen and isolate(s) | No. of repeats at VNTR locus: | | | | | | Isolates within specimen with categorical differences at three loci |
|---|---|---|---|---|---|---|---|
| | A6Cd | B7Cd | C6Cd | E7Cd | G8Cd | CDR60 | |
| 1 | | | | | | | |
| a | 39 | 18 | 45 | 10 | 15 | 10 | a and b at loci A6Cd, B7Cd, and C6Cd |
| b | 37 | 17 | 53 | 10 | 15 | 10 | |
| c, e | 38 | 18 | 46 | 10 | 15 | 10 | |
| d | 38 | 18 | 45 | 10 | 15 | 10 | |
| 3 | | | | | | | |
| a | 18 | 7 | 17 | 12 | 15 | 10 | c and e at loci A6Cd, C6Cd, and G8Cd |
| b | 41 | 8 | 17 | 12 | 15 | 10 | |
| c | 40 | 7 | 17 | 12 | 16 | 10 | |
| d | 42 | 7 | 17 | 12 | 15 | 10 | |
| e | 31 | 7 | 18 | 12 | 15 | 10 | |
| 28 | | | | | | | |
| a, e | 38 | 22 | 22 | 12 | 15 | 10 | a, e and b at loci A6Cd, B7Cd, and G8Cd |
| b | 37 | 21 | 22 | 12 | 16 | 10 | |
| c | 37 | 22 | 23 | 12 | 15 | 10 | |
| d | 37 | 22 | 22 | 12 | 15 | 10 | |
| 29 | | | | | | | |
| a, b | 38 | 21 | 21 | 12 | 16 | 10 | d and e at loci A6Cd, B7Cd, and C6Cd |
| c | 38 | 20 | 21 | 12 | 16 | 10 | |
| d | 38 | 20 | 20 | 12 | 16 | 10 | |
| e | 39 | 21 | 21 | 12 | 16 | 10 | |
| 35 | | | | | | | |
| a | 34 | 20 | 20 | 12 | 17 | 10 | a and c at loci A6Cd, B7Cd, and C6Cd |
| b | 35 | 21 | 20 | 12 | 17 | 10 | |
| c | 35 | 21 | 19 | 12 | 17 | 10 | |
| d, e | 34 | 21 | 20 | 12 | 17 | 10 | |

difference (STRD) from all loci and associate MLVA types with the smallest STRDs. It has been suggested that *C. difficile* isolates with an STRD of two or fewer should be considered "clonal" (1, 4, 7, 14). We applied this method to compare MLVA profiles within each study specimen. Thirty-four specimens (87%) were found to contain isolates which were indistinguishable or had an STRD of no more than two from another isolate from the same specimen. Five specimens (13%) contained at least one isolate which had an STRD of five or more from the next most closely related isolate (Fig. 1).

It is a disadvantage of the Manhattan coefficient that if several repeats at a locus are deleted or duplicated simultaneously, the resulting STRD is large and similarities are obscured.

For this reason, we made alternative comparisons of isolate MLVA profiles within each specimen using the categorical coefficient which associates MLVA types with the smallest number of VNTR locus variants. This revealed that 5 (13%) out of 39 specimens contained isolates differing from each other at three out of six loci (Table 1). Two specimens (1 and 3) contain isolates that differ by both analyses.

The possibility of multistrain acquisition cannot be discounted as an explanation for the variation within specimens seen in this study. However, it was observed that the incidence of tandem-repeat number difference between isolates from one specimen was greater at some loci, being seen in A6Cd, C6Cd, B7Cd, and G8Cd in 20, 16, 7, and 3 out of 39 specimens, respectively, but never at loci E7Cd and CDR60. The stability of some loci within specimens, while still observed to be variable between specimens, suggests that rapid evolution of some loci within the host is a plausible explanation for the intraspecimen variation seen.

This study reinforces observations that the current MLVA schemes for *C. difficile* may be too discriminatory (3, 8). The MLVA profile variations we observed in some specimens could potentially obscure epidemiological links, depending on which isolates are picked from the primary culture. If investigating clusters of CDI, it may be necessary to MLVA type more than one isolate from a specimen to ensure that true epidemiological links are not missed.

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We have no dual or conflicting interests.

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